

# Dihydropyridine-sensitive $\text{Ca}^{2+}$ channel in aneurally cultured human muscles

## Relationship between high-affinity binding site and inhibition of calcium uptake

Claude Desnuelle, Valerie Askanas and W. King Engel

*USC Neuromuscular Center, 637 South Lucas Avenue, Los Angeles, CA 90017, USA*

Received 14 December 1987; revised version received 27 January 1988

Dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels and the relationship between binding of dihydropyridine derivatives and depolarization-induced  $\text{Ca}^{2+}$  uptake have been studied in aneurally cultured human muscle. Analysis of the equilibrium binding of the 1,4-dihydropyridine derivative (+)-PN200-110 revealed a single high-affinity binding site with a  $K_d$  of  $0.15 \pm 0.05$  nM and a  $B_{\text{max}}$  of  $87 \pm 12$  fmol/mg protein. Inhibition of (+)-[ $^3\text{H}$ ]PN200-110 binding by nitrendipine revealed a  $K_i$  of 0.8 nM for the nitrendipine-receptor complex. Depolarization of cultured human muscle achieved by elevating the  $\text{K}^+$  concentration increased the uptake  $^{45}\text{Ca}^{2+}$  which was inhibited by nitrendipine with an  $\text{IC}_{50}$  of 1.1 nM. This study demonstrates that aneurally cultured human muscle has dihydropyridine-sensitive voltage-dependent  $\text{Ca}^{2+}$  channels which are functional when the fibers are depolarized.

$\text{Ca}^{2+}$  channel; Dihydropyridine receptor; Muscle culture

### 1. INTRODUCTION

Dihydropyridine derivatives such as nitrendipine or PN200-110, a subgroup of  $\text{Ca}^{2+}$  antagonists, have been widely used to identify and characterize  $\text{Ca}^{2+}$  channels in several tissues. They are known to bind to one class of voltage-activated  $\text{Ca}^{2+}$  channels in the cell membrane [1]. Voltage-dependent  $\text{Ca}^{2+}$  channels and 1,4-dihydropyridine receptors are found in many tissues but skeletal muscle has the highest density [2]. Dihydropyridine receptors are present on the external membranes of adult human muscle [3]. In animal skeletal muscle, voltage-dependent  $\text{Ca}^{2+}$  channels are assumed to be localized in the transverse-tubular membrane (t-tubules) [4,5].

Developmental aspects of 1,4-dihydropyridine receptors have been previously studied in embryonic chick and rat muscle developing both in tissue culture [6] and in vivo [7]. Biochemical studies of voltage-dependent  $\text{Ca}^{2+}$  channels in developing human muscle have not been reported. Since human fetal material is virtually unavailable for this kind of systematic developmental study, tissue culture of an adult human muscle provides the only suitable model for this kind of investigation. Aneurial tissue cultures of diseased adult human muscle have proved to be important as a tool for studying the pathogenesis of neuromuscular disorders [8], and tissue cultures of normal human muscle provided important information regarding various aspects of human muscle development [9,10].

Here, we describe the properties of 1,4-dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels and the relationship between binding of 1,4-dihydropyridine derivatives and depolarization-induced

*Correspondence (present) address:* C. Desnuelle, Clinique des Maladies Neuromusculaires, CHU La Timone, 13385 Marseille Cedex 5, France

Ca<sup>2+</sup> uptake in aneurally cultured normal human muscle.

## 2. MATERIALS AND METHODS

### 2.1. Cell cultures

Cultures were established from muscle biopsies of patients with no evidence of intrinsic muscle cell disorders after all diagnostic tests were performed. Muscle cultures were established according to our explant re-explantation technique [11]. In brief about 7–10 days after explantation the original explants were removed and outgrowing cells were trypsinized, and plated in gelatin-coated 35-mm petri dishes at a concentration of  $1 \times 10^6$  cells per dish. The cells were grown until fusion in a mixture of Dulbecco's modified Eagle's medium and medium 199 (Gibco, Santa Clara, CA) (3:1, v/v) supplemented with 10% fetal bovine serum (Hy-Clone, Logan, UT), insulin (Sigma, St. Louis, MO) (10  $\mu$ g/ml), epidermal growth factor (Collaborative Research, Lexington, MA) (10 ng/ml) and fibroblast growth factor (25 ng/ml). Immediately after fusion (5–7 days after initiation of cultures) insulin and the growth factors were removed, and the cells continued to grow for 18–21 days in the medium supplemented only with 10% fetal bovine serum. Ca<sup>2+</sup>-uptake experiments and electrophysiologic studies were performed directly in the 35-mm petri dishes. For binding experiments, cells were harvested and homogenized by sonication (Branson sonifier, set 6, 10 s) in Dulbecco's phosphate-buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Gibco) (3–5 mg protein/ml) and the homogenates were stored at  $-80^\circ\text{C}$  until use.

### 2.2. Dihydropyridine-binding assays

Dihydropyridine-binding sites were assayed as in [3] using (+)-[methyl-<sup>3</sup>H]PN200-110 (New England Nuclear, Boston, MA; 70–85 Ci/mmol). Cell homogenate aliquots (0.1–0.2 mg protein) were incubated for 1 h at  $25^\circ\text{C}$  in 1 ml of 50 mM Tris-Cl buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride, in the presence of increasing concentrations of radioligand from 0.05 to 2 nM. At the end of incubation, 400- $\mu$ l aliquots were filtered in duplicate under vacuum on Whatman glass-fiber filters (GF/B) with extensive washing with 50 mM Tris-Cl buffer, pH 7.4. Radioactivity bound to filters was measured using Biofluor (New England Nuclear) in a Packard 2660 scintillation spectrometer with dpm correction. Saturation-isotherm data were analyzed by a software package (Lundon Software, Cleveland, OH) that sequentially examines the experimental data for 1- and 2-site binding models and statistically determines the best fit and most reliable estimates of binding parameters (standard errors of parameter estimates, sums of squares error of the fitted curve, 'run test' to detect the presence of non-randomness in the data and 'F' test to compare the goodness of fit between models).

Competition-isotherm experiments between labeled (+)-PN200-110 and unlabeled nitrendipine were performed on homogenate aliquots (0.1–0.2 mg protein) incubated under the same conditions as for saturation experiments. A fixed concentration of (+)-[<sup>3</sup>H]PN200-110 (1–1.5 nM) and increasing concentrations of nitrendipine (from 0.1 nM to 1  $\mu$ M) were used. The ability of nitrendipine to inhibit specific (+)-[<sup>3</sup>H]PN200-110 binding to its receptor was characterized by the IC<sub>50</sub> value, i.e., the concentration of the compound which in-

hibits binding by 50% under the particular experimental conditions. The overall apparent dissociation constant  $K_i$  of the inhibition for (+)-[<sup>3</sup>H]PN200-110 binding by nitrendipine and the Hill transformation were calculated as described [12].

### 2.3. Ca<sup>2+</sup>-flux experiments

Ca<sup>2+</sup>-uptake studies were performed using the general principles described [6]. The amount of accumulated <sup>45</sup>Ca<sup>2+</sup> was determined in plated cells at  $37^\circ\text{C}$  in 25 mM Hepes-Tris buffer (pH 7.3) containing 5 mM glucose (buffer 1) supplemented with 1.8 mM CaCl<sub>2</sub>, 3  $\mu$ Ci/ml <sup>45</sup>Ca<sup>2+</sup> (NEN). Experiments were conducted in either high K<sup>+</sup> (40 mM KCl-105 mM NaCl; depolarized condition) or low K<sup>+</sup> concentration (5 mM KCl-140 mM NaCl; polarized condition). Time course experiments were performed at  $37^\circ\text{C}$  as follows: (i) cultures were preincubated for 15 min in buffer 1 containing either high or low K<sup>+</sup>; (ii) <sup>45</sup>Ca<sup>2+</sup> uptake was measured on cells maintained in the corresponding incubation media (40 or 5 mM K<sup>+</sup>), supplemented with cold and labeled Ca<sup>2+</sup>, in the absence or presence of 2  $\mu$ M nitrendipine. The time course of <sup>45</sup>Ca<sup>2+</sup> uptake was studied by removing the medium by aspiration at different time intervals between 0 and 4 min. Dose-response curves for nitrendipine inhibition of <sup>45</sup>Ca<sup>2+</sup> uptake were also determined after a 15-min preincubation in buffer 1 but containing high K<sup>+</sup> concentration, using a fixed incubation time (30 s) and in the presence of increasing concentrations of nitrendipine from 0.1 nM to 10  $\mu$ M. In both cases, for time course and dose-response experiments, at the end of incubation, cells were washed three times with 2 ml buffer 1 containing 140 mM choline-chloride and 1 mM La<sup>3+</sup>. After the third wash 2 ml of 0.1 N NaOH was added to each dish and left for 2 h. The radioactivity incorporated by the cells was counted in Aquassure (NEN) in a scintillation spectrometer with dpm correction. All measurements were done in duplicates.

All experiments using dihydropyridine derivatives were performed in subdued lighting because of the light sensitivity of these compounds. Protein determinations were performed according to [13].

### 2.4. Electrophysiology

Electrophysiologic studies were conducted (Dr T. Kobayashi) on cultured human muscle as in [14]. 18–21-day-old myotubes from different culture sets ( $n = 10$ ) were studied at  $35^\circ\text{C}$  using an inverted microscope and microelectrodes filled with 3 M KCl and having a resistance of between 15 and 20 M $\Omega$  for intracellular recording. Permanent record of the resting membrane potential (RMP) was monitored after commencing incubation of the culture under the condition defined as low K<sup>+</sup> concentration (5 mM) for uptake experiments. K<sup>+</sup> concentration was then progressively increased to 40 mM by infusion of the culture dish. After RMP plateaued, washout with low K<sup>+</sup> concentration medium was checked for reversion to the baseline.

## 3. RESULTS

### 3.1. Dihydropyridine receptor in cultured human muscles

The results of the equilibrium saturation assay experiments of (+)-[<sup>3</sup>H]PN200-110 in cultured

human muscle are presented in fig.1. Computer analysis of the data revealed one population of saturable high-affinity binding sites in the range of the radioligand used with a dissociation constant ( $K_d$ ) of  $0.15 \pm 0.05$  nM and a maximal number of binding sites ( $B_{max}$ ) of  $87 \pm 12$  fmol/mg protein (average of 6 separate determinations, from cultured muscle homogenates, each from a different patient biopsy).

Inhibition of (+)-[<sup>3</sup>H]PN200-110 binding by nitrendipine in human muscle cultures is shown in fig.2. In typical experiments, using 0.5–1 nM (+)-[<sup>3</sup>H]PN200-110, non-specific binding, defined as binding in the presence of 2  $\mu$ M nitrendipine, was approx. 50% of total binding. The inhibition of binding appeared monophasic with a Hill slope slightly less than 1.0, indicating that (+)-[<sup>3</sup>H]PN200-110 binding to its muscle receptor was competitively inhibited by nitrendipine. The data obtained from two different experiments with two different culture sets gave a concentration for half-maximal inhibition by nitrendipine of (+)-[<sup>3</sup>H]PN200-110 binding ( $IC_{50}$ ) of 5.2 nM. The  $K_i$  for the nitrendipine-receptor complex calculated from the  $IC_{50}$  value was 0.8 nM, similar to the  $K_d$  values obtained in adult human muscle homogenate using tritiated nitrendipine [15] and cultured embryonic chick muscle [6] (0.5 and 0.4 nM, respectively).

### 3.2. Nitrendipine-sensitive <sup>45</sup>Ca<sup>2+</sup> uptake in cultured human muscle

The influence of nitrendipine on <sup>45</sup>Ca<sup>2+</sup> uptake in cultured human muscle was analyzed in high and low K<sup>+</sup> buffers. An increase in K<sup>+</sup> concentration in the incubation buffer from 5 to 40 mM resulted in increasing accumulation of <sup>45</sup>Ca<sup>2+</sup> in cultured human muscle cells (fig.3) caused by depolarization of the muscle fibers by the high K<sup>+</sup> concentration. In our cultured human muscle membrane potential changed from  $-53 \pm 6$  to  $-26 \pm 4$  mV when the K<sup>+</sup> concentration rose from 5 to 40 mM. K<sup>+</sup>-stimulated, time-dependent, <sup>45</sup>Ca<sup>2+</sup> uptake was rapid, being half-maximal within 30 s. The initial rate of <sup>45</sup>Ca<sup>2+</sup> accumulation in 40 mM K<sup>+</sup> was approx. 4-times that measured in 5 mM K<sup>+</sup>. Extrapolated from the initial rates of uptake, more than 1 nmol <sup>45</sup>Ca<sup>2+</sup>/min per mg protein could be transported under the high K<sup>+</sup> condition vs only 0.3 nmol/min per mg protein

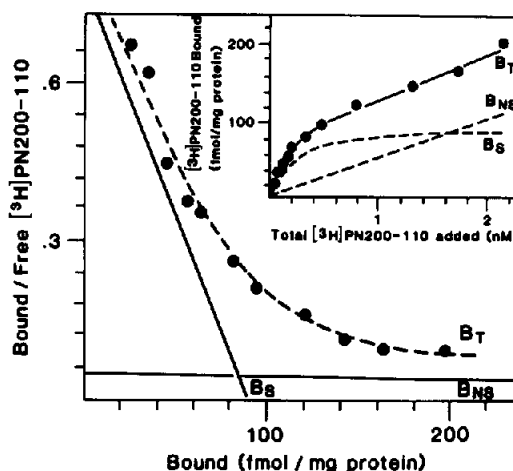


Fig.1. Typical example of binding of (+)-[methyl-<sup>3</sup>H]PN200-110 to human cultured muscle homogenates. Experiment was carried out as described in section 2. Protein concentration was 0.13 mg/ml. (Inset) Total ( $B_T$ ) (+)-[<sup>3</sup>H]PN200-110 binding (●) was fitted by the computerized analysis with a non-linear model. Non-specific binding ( $B_{NS}$ ) and specific binding ( $B_S$ ), reported as broken lines, were calculated from the total binding data. The main figure shows a Scatchard representation from the saturation isotherm data for total binding ( $B_T$ ). The saturable specific binding ( $B_S$ ) was calculated after subtraction of the non-saturable binding ( $B_{NS}$ ) participating. In this typical experiment  $K_d = 0.165$  nM and  $B_{max} = 82$  fmol/mg protein.

in the case of the low K<sup>+</sup> condition. K<sup>+</sup>-stimulated <sup>45</sup>Ca<sup>2+</sup> uptake plateaued in approx. 2 min, after which the rate of <sup>45</sup>Ca<sup>2+</sup> accumulation in 40 mM K<sup>+</sup> paralleled the rate observed in 5 mM K<sup>+</sup>. The depolarization-induced <sup>45</sup>Ca<sup>2+</sup> uptake was not dependent upon extracellular Na<sup>+</sup> concentration and thus was not due to Na<sup>+</sup>/Ca<sup>2+</sup> exchange, since uptake could be demonstrated when NaCl was replaced by choline in the incubation buffer. The time-dependent <sup>45</sup>Ca<sup>2+</sup> uptake induced by K<sup>+</sup> was inhibited by 2  $\mu$ M nitrendipine (fig.3). The dose-response curve for the effect of nitrendipine on <sup>45</sup>Ca<sup>2+</sup> uptake is shown in the inset to fig.3. When cells were incubated under depolarizing conditions for 30 s with increasing concentrations of nitrendipine, the nitrendipine-sensitive rates of <sup>45</sup>Ca<sup>2+</sup> uptake were obtained by subtracting from the initial rates of <sup>45</sup>Ca<sup>2+</sup> uptake the values obtained under the same conditions in the presence of 2  $\mu$ M nitrendipine. The concentration for half-maximal inhibition of the <sup>45</sup>Ca<sup>2+</sup> uptake was 1.1 nM (two

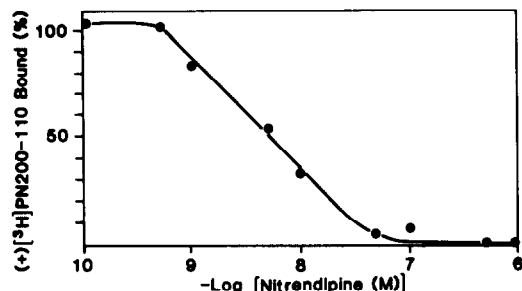


Fig.2. Inhibition of specific (+)-[methyl- $^3\text{H}$ ]PN200-110 (76 Ci/mmol) binding in human cultured muscle homogenate by increasing concentrations of unlabeled nitrendipine measured under equilibrium conditions at  $25^\circ\text{C}$  and expressed as percent of maximum specific binding (100% = absence of nitrendipine). In this typical example, using 1.2 nM (+)-[ $^3\text{H}$ ]PN200-110, half displacement was observed at 5.1 nM. Determined in parallel on the same preparation by direct binding  $K_d$  was 0.20 nM. Data in the text are means from two different culture sets, each assay in duplicate.

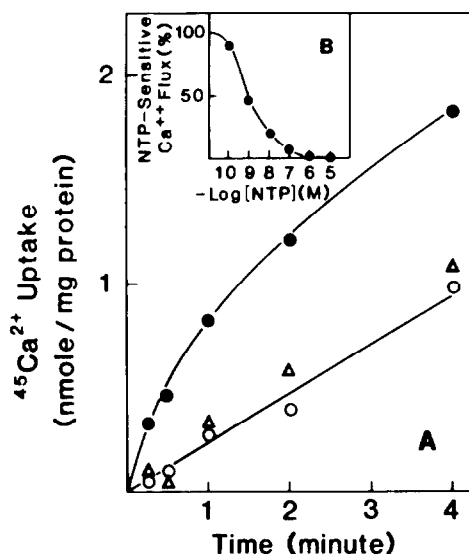


Fig.3.  $\text{Ca}^{2+}$  flux experiments. Time course of  $^{45}\text{Ca}^{2+}$  accumulation into human muscle in culture. Uptake of  $^{45}\text{Ca}^{2+}$  in buffer containing 40 mM  $\text{K}^+$  (●), 5 mM  $\text{K}^+$  (○) and 40 mM  $\text{K}^+$  in the presence of  $2\ \mu\text{M}$  nitrendipine ( $\Delta$ ) were determined for the incubation times indicated as described in section 2. (Inset) Inhibition of specific nitrendipine-sensitive  $^{45}\text{Ca}^{2+}$  flux in high  $\text{K}^+$  medium. Values indicated are expressed as percent of control of  $\text{K}^+$ -stimulated uptake for incubation in high  $\text{K}^+$  medium in the absence of nitrendipine. Specific nitrendipine-sensitive  $^{45}\text{Ca}^{2+}$  accumulation was defined as the uptake obtained in the presence of  $2\ \mu\text{M}$  nitrendipine. Data are averages of two separate experiments from different sets of culture each conducted in duplicate.

experiments in duplicate from two different sets of muscle cultures).

#### 4. DISCUSSION

The present findings in aneural human muscle cultures include demonstration of: (i) a high-affinity binding site for 1,4-dihydropyridine derivatives, (ii)  $\text{K}^+$  depolarization-induced  $^{45}\text{Ca}^{2+}$  uptake and (iii) a correlation between the binding affinity of the 1,4-dihydropyridine-receptor complex and direct inhibition by 1,4-dihydropyridine of  $^{45}\text{Ca}^{2+}$  uptake under depolarizing condition.

We found that (+)-PN200-110 binds to a specific and saturable high-affinity site ( $K_d$  0.15 nM) in cultured human skeletal muscle. This binding was competitively inhibited by nanomolar nitrendipine, indicating that nitrendipine binds with high affinity to this receptor ( $K_i$  0.8 nM). Similar high-affinity binding was found for (+)-[ $^3\text{H}$ ]PN200-110 and [ $^3\text{H}$ ]nitrendipine binding to the 1,4-dihydropyridine receptor of surface membranes isolated from adult human skeletal muscle [3] and in crude homogenates from adult human muscle [14] (0.2 and 0.5 nM, respectively). In contrast with adult muscle, the 1,4-dihydropyridine receptor concentration in aneurally cultured human muscle is low (87 fmol/mg protein in culture vs 460 fmol/mg protein to 7 pmol/mg protein in adult human muscle depending on the degree of purification of membranes) (cf. [3,15]).

Biochemical and electrophysiologic data have shown that in adult animal muscle voltage-dependent  $\text{Ca}^{2+}$  channels are mainly localized in the transverse tubular system [16,17]. Since cultured human muscle fiber grown aneurally under the present conditions do not have well organized t-tubules, the localization of the 1,4-dihydropyridine receptor in this cultured muscle remains obscure. One possible explanation is that an unorganized primitive tubular system existing in aneurally cultured muscle (presumably pre-t-tubule structures) contains voltage-dependent  $\text{Ca}^{2+}$  channels. However, the possible existence of voltage-dependent  $\text{Ca}^{2+}$  channels in other structures cannot be ruled out.

Electrophysiologic studies of human muscle cultured for 8–15 days by others under different conditions failed to demonstrate  $\text{Ca}^{2+}$  conductance

[18], suggesting the absence of a voltage-dependent  $\text{Ca}^{2+}$  channel in this cell. Whether this obvious difference between their studies and ours reflects the difference in sensitivity between electrophysiological and biochemical analysis of voltage-dependent  $\text{Ca}^{2+}$  channels or a different stage of maturation of human muscle cultured under different conditions cannot be answered as yet.

In the present study, in aneurally cultured muscle the  $\text{Ca}^{2+}$  channel function is analyzed in terms of depolarization-induced  $\text{Ca}^{2+}$  uptake. Depolarization of muscle culture by high  $\text{K}^+$  induces a greater  $^{45}\text{Ca}^{2+}$  flux than under polarized conditions in low  $\text{K}^+$ . This depolarization-induced  $\text{Ca}^{2+}$  flux is almost completely eliminated in the presence of  $2\text{ }\mu\text{M}$  nitrendipine. Similar observations have been made with a clonal pheochromocytoma cell line (PC 12) in culture [19,20], primary cultures of cerebellar granule cells [21] and primary cultures of chick embryo muscle [6]. When compared with those tissues, the time course of accumulation of  $^{45}\text{Ca}^{2+}$  in primary cultures of human muscle is longer and the ability to accumulate  $\text{Ca}^{2+}$  5–20-times lower. The low concentration of voltage-dependent  $\text{Ca}^{2+}$  channels in this system demonstrated by binding studies could explain such observations.

In intact frog sartorius muscle the affinity constant for the complex formed by 1,4-dihydropyridine with its receptor was found to be 100-times lower than the half-maximal concentration of 1,4-dihydropyridine needed to block muscle contraction in this particular preparation [22]. Therefore, it was postulated that the 1,4-dihydropyridine receptor in muscle was not the functional  $\text{Ca}^{2+}$  channel. Our biochemical and pharmacokinetic studies of cultured human muscle demonstrate that (i) 1,4-dihydropyridine derivatives bind to a single class of saturable high-affinity sites in cell homogenates and (ii) half-maximal blockade by nitrendipine of  $^{45}\text{Ca}^{2+}$  flux measured in high  $\text{K}^+$  concentration on intact cells is in a similar nanomolar range. These findings suggest that under depolarized conditions, inhibition of  $^{45}\text{Ca}^{2+}$  uptake by 1,4-dihydropyridine may be functionally linked to this high-affinity dihydropyridine-binding site. These results are in agreement with electrophysiological studies in animal cardiac [23] and skeletal muscle [24] which demonstrated that the high-affinity 1,4-dihydropyridine-binding site

and the inactivated muscle voltage-dependent  $\text{Ca}^{2+}$  channel were identical.

In conclusion, the close relationship between the biochemically determined and the functional properties of the dihydropyridine-receptor complex has been demonstrated in aneurally cultured human skeletal muscle using 1,4-dihydropyridine binding and  $\text{Ca}^{2+}$ -flux techniques. These data, in agreement with the previously described role of the 1,4-dihydropyridine receptor, may serve as a basis for studying voltage-dependent  $\text{Ca}^{2+}$  channels in cultured human muscle from patients with neuromuscular diseases in which membrane abnormality is suspected. Further biochemical studies of voltage-dependent  $\text{Ca}^{2+}$  channels in our new culture system, in which human muscle cultured in monolayers is functionally innervated by fetal rat spinal cord and has well developed t-tubules [25,26], will be of importance in evaluating the influence of neural factors on the developmental properties of voltage-dependent  $\text{Ca}^{2+}$  channels.

*Acknowledgements:* This work was supported by grants from the Muscular Dystrophy Association, the CNRS-Département des Sciences de la Vie, and the Association des Myopathes de France. C.D. was a W. Donham and Coleen Crawford Postdoctoral Research Fellow. Ghislaine Gallez-Hawkins provided technical assistance in tissue culture. We thank Dr Scriabine (Miles Institute for Preclinical Pharmacology) for providing nitrendipine.

## REFERENCES

- [1] Janis, R.A. and Scriabine, A. (1983) *Biochem. Pharmacol.* 32, 3499–3507.
- [2] Gould, R.J., Murphy, K.M. and Snyder, S.H. (1984) *Mol. Pharmacol.* 25, 235–241.
- [3] Desnuelle, C., Liot, D., Serratrice, G. and Lombet, A. (1985) *FEBS Lett.* 188, 222–226.
- [4] Almers, W. and Palade, P.T. (1981) *J. Physiol.* 312, 159–176.
- [5] Fosset, M., Jaimovich, E., Delpont, E. and Lazdunski, M. (1983) *J. Biol. Chem.* 258, 6086–6092.
- [6] Schmid, A., Renaud, J.F., Fosset, M., Meaux, J.P. and Lazdunski, M. (1984) *J. Biol. Chem.* 259, 11366–11372.
- [7] Schmid, A., Kazazoglou, T., Renaud, J.F. and Lazdunski, M. (1984) *FEBS Lett.* 172, 114–118.
- [8] Askanas, V. (1984) in: *Neuromuscular Diseases* (Serratrice, G. et al. eds) pp.373–379, Raven, New York.
- [9] Askanas, V. and Engel, W.K. (1979) in: *Handbook of Clinical Neurology* (Vinken, P.J. et al. eds) vol.40, pp.155–164, North-Holland, Amsterdam.
- [10] Miranda, A.F., Somer, H. and Di Mauro, S. (1979) in: *Muscle Regeneration* (Mauro, A. ed.) pp.453–473, Raven, New York.

- [11] Askanas, V. and Engel, W.K. (1975) *Neurology* 25, 58–67.
- [12] Lombet, A., Renaud, J.F., Chicheportiche, R. and Lazdunski, M. (1981) *Biochemistry* 20, 1279–1285.
- [13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) *J. Biol. Chem.* 193, 265–275.
- [14] Tamoush, A.J., Askanas, V., Nelson, P.G. and Engel, W.K. (1983) *Neurology* 33, 311–316.
- [15] Desnuelle, C., Renaud, J.F., Delpont, E., Serratrice, G. and Lazdunski, M. (1986) *Muscle Nerve* 9, 148–151.
- [16] Almers, W., Fink, R. and Palade, P.T. (1981) *J. Physiol.* 312, 117–207.
- [17] Jaimovich, E., Donoso, P., Liberona, J.L. and Hidalgo, C. (1986) *Biochim. Biophys. Acta* 855, 89–98.
- [18] Trautmann, A., Delaporte, C. and Marty, A. (1986) *Pflüger Arch.* 406, 163–172.
- [19] Ritchie, A.K. (1979) *J. Physiol.* 286, 541–561.
- [20] Toll, L. (1982) *J. Biol. Chem.* 257, 11189–11192.
- [21] Carboni, E., Wojcik, W.J. and Costa, E. (1985) *Neuropharmacology* 24, 1123–1126.
- [22] Schwartz, L.M., McCleskey, E.W. and Almers, W. (1985) *Nature* 314, 747–751.
- [23] Bean, B.P. (1984) *Proc. Natl. Acad. Sci. USA* 81, 6388–6392.
- [24] Cognard, C., Romey, G., Galizzi, J.P., Fosset, M. and Lazdunski, M. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1518–1522.
- [25] Askanas, V., Kwan, H., Alvarez, R., Kobayashi, T., Martinuzzi, A. and Engel, W.K. (1986) *Muscle Nerve* 9, 5S, 13.
- [26] Kobayashi, T. and Askanas, V. (1985) *Exp. Neurol.* 88, 327–335.